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# Ethanol Production by Thermophilic Bacteria: Relationship Between Fermentation Product Yields of and Catabolic Enzyme Activities in *Clostridium thermocellum* and *Thermoanaerobium brockii*

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Significant quantitative differences in end-product yields by two strains of *Clostridium thermocellum* and one strain of *Thermoanaerobium brockii* were observed during cellobiose fermentation. Most notably, the ethanol/$H_2$ and lactate/acetate ratios were drastically higher for *T. brockii* as compared with *C. thermocellum* strains LQRI and AS39. Exogenous $H_2$ addition (0.4 to 1.0 atm) during culture growth increased the ethanol/acetate ratio of both *T. brockii* and AS39 but had no effect on LQRI. All strains had an operative Embden-Meyerhof glycolytic pathway and displayed catabolic activities of fructose-1,6-diphosphate-activated lactate dehydrogenase, coenzyme A acetylating pyruvate and acetaldehyde dehydrogenase, hydrogenase, ethanol dehydrogenase, and acetate kinase. Enzyme kinetic properties (apparent $K_m$, $V_{max}$, and $Q_{10}$ values) and the specificity of electron donors/acceptors for different oxidoreductases involved in pyruvate conversion to fermentation products were compared in the three strains. Both species contained ferredoxin-linked pyruvate dehydrogenase and pyridine nucleotide oxidoreductases. Ferredoxin-nicotinamide adenine dinucleotide (NAD) reductase activity was significantly higher in *T. brockii* than in AS39 and was not detectable in LQRI. $H_2$ production and hydrogenase activity were inversely related to ferredoxin-NAD reductase activity in the three strains. Ferredoxin-NAD phosphate reductase activity was present in cell extracts of both species. Alcohol dehydrogenase activity in *C. thermocellum* was NAD dependent, unidirectional, and inhibited by low concentrations of NAD and ethanol. Ethanol dehydrogenase activity of *T. brockii* was both NAD and NADP linked, reversible, and not inhibited by low levels of reaction products. The high lactate yield of *T. brockii* correlated with increased fructose-1,6-diphosphate. The relation of catabolic enzyme activity and quantitative differences in intracellular electron flow and fermentation product yields of these thermophilic bacteria is discussed.

The production of ethanol from microbial biomass fermentations has generated considerable research interest (5, 19, 21). Several factors account for technological interest in thermophilic ethanologenic fermentations: direct conversion of delignified biomass polymers, fermentation of both hexoses and pentoses, high metabolic rates, physically (i.e., thermally stable) and chemically stable enzymes and cells, and facilitated end-product recovery (24). Thus, several thermophilic, ethanologenic species (i.e., *Clostridium thermocellum, C. thermohydrosulfuricum,* and *Thermoanaerobium brockii*) have been suggested as potentially useful for bioethanol production (24). Nonetheless, considerably more knowledge on the catabolic pathways and the regulation of end-product formation is required for optimal utilization of these anaerobic bacterial species in industrial fermentations.

*C. thermocellum* and *T. brockii* form similar end products (ethanol, $H_2/CO_2$, lactate, and acetate) from fermentation of cellulose and starch, respectively (20, 26). Considerable variations in physiological features, including the range of energy sources metabolized, fermentation products produced, and enzyme activities detected, have been reported in different *C. thermocellum* strains (10, 14-18, 20, 24). Patni and Alexander (15) concluded that *C. thermocellum* catabolized glucose via the Embden-Meyerhof pathway on the basis of identifying key enzyme activities including fructose-1,6-diphosphate (FDP) aldolase and glucokinase. We recently demonstrated that the glucose fermentation route of *T. brockii* also involved the Embden-Meyerhof pathway (9).

The enzymes associated with the conversion of pyruvate to saccharide fermentation products

have been more thoroughly investigated in *T. brockii* than in *C. thermocellum*. Hydrogenase, pyruvate dehydrogenase, FDP-activated lactate dehydrogenase, acetaldehyde dehydrogenase, ethanol dehydrogenase, and acetate kinase were detected in *T. brockii* cell extracts (9), whereas only a non-FDP-activated lactate dehydrogenase was reported in *C. thermocellum* (15).

In the present report the different yields of end products formed during cellobiose fermentation by strains of *C. thermocellum* and *T. brockii* are explained by understanding subtle differences in the activity and direction of catabolic enzymes involved in intraspecies electron flow.

## MATERIALS AND METHODS

**Chemicals.** All chemicals were reagent grade. Enzymes and coenzymes were obtained from Sigma Chemical Co. (St. Louis, Mo.); cellulose MN-300 was from Machery, Nagel and Co. (Duren, West Germany); $N_2$, $H_2$, and He gases were purchased from Matheson Gas Products, (Joliet, Ill.) and were passed through heated (310°C) copper filings to remove traces of $O_2$. Purified ferredoxin from *C. pasteurianum* was kindly donated by L. Mortensen, Purdue University, West Lafayette, Ind.

**Organisms.** *T. brockii* neotype strain HTD4 (26) and two strains of *C. thermocellum* obtained from different sources were used. *C. thermocellum* strain LQRI was isolated from a contaminated culture of *C. thermocellum* strain LQ8 originally obtained from R. Quinn, University of Iowa. Strain LQRI differed significantly from LQ8 (14, 20) in that this strain fermented glucose in complex medium as energy source, did not produce butyrate as an end product, and produced extracellular carboxymethylcellulase when grown on cellobiose or glucose. *C. thermocellum* strain AS39 was obtained from A. Demain, Massachusetts Institute of Technology; it is a mutant strain obtained from *C. thermocellum* ATCC 27405 (American Type Culture Collection, Rockville, Md.) that displayed increased cellulase activity (18). Both AS30 and LQRI have similar substrate ranges for growth, but AS39 rarely sporulates and appears microscopically as shorter and thicker rods. *C. pasteurianum* was obtained from the culture collection of the Department of Bacteriology, University of Wisconsin.

**Media and cultivation conditions.** Fermentation studies employed a cellobiose complex medium (CC medium) that contained, per liter of distilled water: $KH_2PO_4$, 1.5 g; $K_2HPO_4$, 2.9 g; $MgCl_2 \cdot 6H_2O$, 0.5 g; urea, 2.14 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; $FeSO_4 \cdot 6H_2O$, 1.25 mg; morpholinepropanesulfonic acid, 10 g; cellobiose (or cellulose MN-300, or glucose), 8 g; yeast extract, 6 g; cysteine-HCl, 1 g; and resazurin, 2 mg. The pH was adjusted to 7.0 with NaOH. The gas phase was $N_2$. Cells were cultured in 24-ml anaerobic culture tubes (18 by 142 mm) obtained from Bellco Glass, Inc. (Vineland, N.J.) that contained 10 ml of medium and were sealed with no. 1 butyl-rubber stoppers. Test tube cultures were incubated at 60°C (*C. thermocellum*) or 65°C (*T. brockii*) without shaking.

Cells used for analysis of enzyme activities were grown in 5-liter New Brunswick fermentors which contained 3.5 liters of CC medium for growth of *C. thermocellum* and *T. brockii* on cellobiose and TYEG medium (9) for growth of *T. brockii* and *C. pasteurianum* on glucose as energy source. Fermentor cultures were maintained with constant stirring (100 rpm) and continuous $N_2$ gassing (20 $cm^3$/min) at 60°C for *C. thermocellum*, at 65°C for *T. brockii*, and at 37°C for *C. pasteurianum*. Cells were harvested in late exponential growth phase and collected by centrifuging at 35,000 × *g* in a Sorvall RC-5 centrifuge (DuPont Instruments) equipped with a KSB continuous-flow system.

**Metabolic characterization.** All growth and metabolic experiments employed duplicate or more anaerobic culture tubes, and individual experiments were duplicated or triplicated. Growth was determined by quantification of absorbance at 540 nm or by cell dry weight. Absorbance was measured directly by insertion of the anaerobic culture tubes into a Spectronic 20 (Bausch & Lomb, Rochester, N.Y.) spectrophotometer. Cell dry weight was determined by filtration of the culture through a 0.45-μm filter (Millipore Corp., Bedford, Mass.) followed by drying at 65°C to a constant weight. Glucose was determined with Statzyme reagent (Worthington Biochemicals Corp., Chicago, Ill.). Both $^{14}C$-labeled and nonradioactive metabolic gases were analyzed by the procedures described by Nelson and Zeikus (13). Organic alcohols and acids were determined as described by Zeikus et al. (26). L-Lactic acid was determined by a standard enzyme assay (2).

**Determination of intracellular levels of FDP.** Cells were grown in tubes or flasks that contained CC medium until the mid-logarithmic phase (absorbance at 540 nm, 0.5 to 0.8). The cultures were then rapidly cooled in acetone-dry ice and centrifuged for 5 min at 35,000 × *g*. The cells were suspended in 10% $HClO_4$ at one-fortieth of the original culture volume and mixed for 10 min. The suspension was neutralized by use of concentrated $K_2CO_3$ as titrant with methyl red indicator and then centrifuged (10,000 × *g*). The concentration of FDP was determined in the supernatant by a standard method (2).

**Preparation of cell extracts.** Anaerobic conditions were maintained throughout the entire procedure, and all manipulations were performed under a helium atmosphere at 4°C. Cells (2 g, wet weight) were placed in a 15-ml Corex tube that contained 8 ml of 25 mM Tris-hydrochloride (pH 7.4), 3 mM dithiothreitol, and 5 μg of DNase. After thorough mixing, the cell suspension was passed through a French pressure cell at 1,400 kg/$cm^2$. The cell lysate was collected in a centrifuge tube, sealed with a flanged rubber bung, and centrifuged at 10,000 × *g* for 30 min. The supernatant was removed with a glass syringe and injected into glass vials that contained helium gas and were sealed with soft rubber bungs. Extracts were used immediately or were stored at −20°C. The protein content of extracts was determined by the method of Bradford (3) with the use of reagents from Bio-Rad Laboratories (Rockville, N.Y.).

**Preparation of ferredoxin-free extracts, crude ferredoxins, and ferredoxin assay.** Ferredoxin-free

extracts were prepared by a standard method based on the high affinity of ferredoxin for DEAE-cellulose (12). Cell extract (1.3 ml, 15 mg/ml) was mixed with 0.5 ml of a 1:1 suspension of wet DEAE-cellulose (Whatman 52) in 0.30 M Tris-hydrochloride, pH 7.3, and 0.3 M NaCl. The anaerobic mixture was shaken for 5 min and then allowed to settle. The supernatant was used for the assays. Crude ferredoxins were prepared by use of the first steps of Mortenson's acetone purification method (11). Extract (1 ml, 15 mg/ml) was mixed with 1 ml of acetone, and the precipitate obtained was removed by centrifugation (10,000 × *g*); 0.3 ml of wet DEAE-cellulose was then added to the supernatant. After mixing for 5 min, the ion exchanger was loaded into a small column and washed with water; ferredoxin-like protein was desorbed by 0.5 M NaCl. The volume of the protein solution collected was 1 ml. Assay of ferredoxins was based on the requirement for ferredoxin for the function of coenzyme A (CoA)-dependent pyruvate dehydrogenase of *C. pasteurianum* (12).

**Enzyme assays.** All assays were performed at 40°C (unless specified in the text) under anaerobic conditions as described by Zeikus et al. (25). All activities were measured by modifications of standard assay methods (2, 25). Specific activities were determined in a range where linearity with protein concentration was established. A unit of enzyme activity represents the amount of enzyme catalyzing the conversion of 1 μmol of substrate per min into specific products. The concentrations of components in the reaction mixtures (1-ml total volume) used for analysis of specific enzymes were as follows:

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)—0.1 M Tris-hydrochloride, pH 7.5, 2.5 mM $MnCl_2$ or 6 mM $MgCl_2$, 2 mM glucose-6-phosphate, 1 mM dithiothreitol, and 1.0 mM NAD(P).

Gluconate-6-phosphate dehydrogenase (EC 1.1.1.43)—as above but with gluconate-6-phosphate replacing glucose-6-phosphate.

FDP aldolase (EC 4.1.2.13)—0.05 M Tris-hydrochloride, pH 7.5, 0.1 mM cysteine-HCl, 0.1 M potassium acetate, 2 mM FDP, 0.7 mM $CoCl_2$, 0.25 mM NADH, 20 U of triose phosphate isomerase, and 2.0 U of glycerol-3-phosphate dehydrogenase.

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)—0.1 mM Tricine-hydrochloride, pH 8.1, 5 mM potassium phosphate, 20 mM neutralized sodium arsenate, 2 mM FDP, 2 mM dithiothreitol, 1 mM NAD, and 1.0 U of aldolase.

Pyruvate dehydrogenase (CoA acetylating) (EC 1.2.7.1)—0.1 M Tris-acetate, pH 7.8, 5 mM pyruvate, 0.1 mM CoA, 7 mM sodium arsenate, and 2 mM methyl viologen.

Lactate dehydrogenase (FDP activated) (EC 1.1.2.3)—0.1 M imidazole-HCl buffer, pH 6.2, 0.25 mM NADH, 10 mM pyruvate, and 1 mM FDP.

Acetaldehyde dehydrogenase (CoA acetylating) (EC 1.2.1.10)—0.1 M Tris-hydrochloride, pH 7.2, 1 mM dithiothreitol, 0.1 mM CoA, 7 mM sodium arsenate, 0.5 mM NAD, 10 mM acetaldehyde, and 0.5 U of phosphotransacetylase.

Hydrogenase (EC 1.12.1.1)—0.1 M Tris-acetate, pH 7.8, 2 mM methyl viologen, 2 mM dithiothreitol, and 1 atm of $H_2$ gas.

Phosphotransacetylase (EC 2.3.1.8)—0.1 M Tris-acetate, pH 7.8, 5 mM pyruvate, 2 mM methyl viologen, and 0.1 mM CoA; 7 mM sodium arsenate was added after initial pyruvate dehydrogenase activity stopped.

Acetate kinase (EC 2.7.2.1)—(a) 0.1 M Tris-hydrochloride, pH 7.2, 3 mM $MgCl_2$, 2 mM glucose, 0.5 mM NADP, 1 U each of hexokinase and glucose-6-phosphate dehydrogenase, 1 mM ADP, and 4 mM acetylphosphate; (b) 0.1 M Tris-hydrochloride, pH 8, 6 mM $MgCl_2$, 2 mM ATP, 1 mM phosphoenolpyruvate, 0.25 mM NADH, 0.2 M potassium acetate, and 2.0 U each of pyruvate kinase and lactate dehydrogenase.

Myokinase (EC 2.7.4.3)—as above (a) with acetyl phosphate omitted.

Adenosine triphosphatase (EC 3.6.1.3)—as above (b) with potassium acetate omitted.

Ethanol dehydrogenase, acetaldehyde reductase (EC 1.1.1.12)—(a) 0.1 M Tris-hydrochloride, pH 7.8, 2 mM dithiothreitol, 1 mM NAD(P)H, and 5 mM acetaldehyde; (b) 0.1 M Tris-hydrochloride, pH 8.5, 0.5 M ethanol, and 0.5 mM NAD(P).

Malic enzyme (EC 1.1.1.40)—0.1 M Tris-hydrochloride, pH 7.0, 5 mM $MnCl_2$, 20 mM $NH_4Cl$, 2 mM L-malate, and 0.5 mM NADP.

Malate dehydrogenase, oxalacetate reductase (EC 1.1.1.37)—0.1 M Tris-hydrochloride, pH 7.0, 5 mM EDTA, 1 mM oxalacetate, and 0.2 mM NADH.

Carboxymethyl cellulase was assayed by the procedure of Weimer and Zeikus (20).

Determination of ferredoxin-NAD(P) oxidoreductases and NADH-linked ferredoxin reductase by measurement of $H_2$ formation from NADH was done by the method of Jungermann et al. (8). Pyridine nucleotide oxidation or reduction reactions were measured at 334 nm ($\epsilon_{334} = 6.10\ mM^{-1}\ cm^{-1}$), and methyl viologen reduction was measured spectrophotometrically at 578 nm ($\epsilon_{578} = 9.78\ mM^{-1}\ cm^{-1}$) with an Eppendorf recording spectrophotometer. The apparent $K_m$ and $V_{max}$ for individual enzymes were calculated from Lineweaver-Burk plots. $Q_{10}$ values were determined from Arrhenius plots. Determinations of apparent product inhibition constants ($K_i$) of NAD and ethanol by *C. thermocellum* alcohol dehydrogenase were obtained from slopes of Dixon plots ($1/v$ versus $[I]$) in the presence of different fixed concentrations of substrate (i.e., acetaldehyde, NAD).

## RESULTS

**Comparison of cellobiose fermentation.** A time course for cellobiose fermentation by *C. thermocellum* strain AS39 is shown in Fig. 1. All end products and cellulase activity were formed in parallel during the fermentation. Conversion of cellobiose to end products continued after growth ceased. A similar relation of end-product formation to growth was observed for cellobiose fermentation by all three strains.

End-product yields of cellobiose fermentations by the three strains are shown in Table 1. The calculated fermentation balances for *C. thermocellum* strains and *T. brockii* were within good experimental error (on the basis of decrease

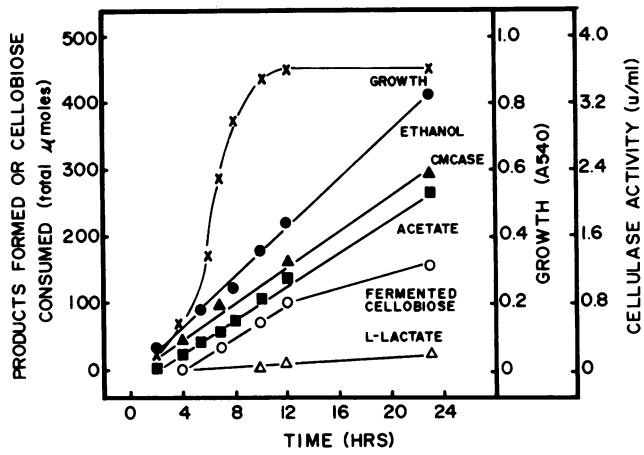


FIG. 1. *Time course of cellobiose fermentation by C. thermocellum AS39. Anaerobic culture tubes contained 10 ml of CC medium and were incubated at 60°C.*

TABLE 1. *Cellobiose fermentation products of C. thermocellum AS39 and LQRI and T. brockii HTD4*[a]

| Product | Yield (total μmol formed/tube) | | |
|---|---|---|---|
| | AS39 | LQRI | HTD4 |
| Ethanol | 230 | 157 | 224 |
| Acetic acid | 110 | 125 | 48 |
| Lactic acid | 12 | 24 | 352 |
| $CO_2$ | 325 | 346 | 230 |
| $H_2$ | 121 | 286 | 20 |
| C recovery | 0.8 | 0.8 | 0.9 |
| O/R index | 0.9 | 0.9 | 1.0 |

[a] Cultures were grown for 18 h in 24-ml tubes that contained 10 ml of CC medium. O/R, Oxidation/reduction.

TABLE 2. *Effect of $H_2$ on the cellobiose fermentation product pattern of C. thermocellum AS39 and LQRI and T. brockii HTD4*[a]

| Product | Yield (total μmol formed/tube) | | | | | |
|---|---|---|---|---|---|---|
| | LQRI | | AS39 | | HTD4 | |
| | $N_2$ | $H_2$ | $N_2$ | $H_2$ | $N_2$ | $H_2$ |
| Ethanol | 120 | 110 | 220 | 300 | 360 | 170 |
| Lactate | 40 | 40 | 60 | 80 | 500 | 210 |
| Acetate | 150 | 130 | 180 | 150 | 80 | 20 |
| Ethanol/acetate | 0.8 | 0.8 | 1.2 | 2.0 | 4.5 | 8.5 |

[a] Cultures were grown in 24-ml tubes that contained 10 ml of modified CC medium with either $N_2$ (1 atm) or $H_2$ (1 atm for *C. thermocellum* and 0.4 atm for *T. brockii*). Cultures were incubated for 20 h (*C. thermocellum*) and 30 h (*T. brockii*) prior to product analysis.

in cellobiose reducing power and the end products formed). The oxidation/reduction index did not indicate formation of additional reduced or oxidized carbon sources or products for the three cultures. The main differences between *C. thermocellum* and *T. brockii* included higher lactate, lower hydrogen, and lower acetate production by *T. brockii*. Differences between *C. thermocellum* strains were more subtle but could be obtained repeatedly under identical fermentation conditions. AS39 always produced lower $H_2$ yields than LQRI. Similar product yields were observed for *C. thermocellum* grown on cellulose and for *T. brockii* grown on glucose.

The effect of added $H_2$ on the fermentation product patterns of the three strains is shown in Table 2. The ratio of ethanol to acetate formed was increased markedly by addition of $H_2$ to HTD4 and AS39 but not to LQRI. The effect of $H_2$ was more pronounced for *C. thermocellum* AS39 at higher $H_2$ pressure. For example, the ethanol/acetate ratio increased from 2.0 to 2.5 when AS39 was grown on cellobiose with an initial $H_2$ pressure of 1.8 atm. Hydrogen noticeably inhibits growth rate and yield of *T. brockii* (Ben-Bassat et al., unpublished data) but not *C. thermocellum* (20).

**Enzymes associated with sugar conversion to pyruvate.** To confirm that *C. thermocellum* used the Embden-Meyerhof glycolytic pathway, we initiated radioactive tracer studies with specifically labeled [$^{14}$C]glucose. The results of experiments that traced the origin of $^{14}CO_2$ from various positions in [$^{14}$C]glucose are shown in Table 3. The specific radioactivity of $CO_2$ evolved was 2.5 to 3.0 times higher when the label resided in 3,4 positions of glucose, as compared with uniformly labeled glucose. No significant $^{14}CO_2$ was obtained from label at position 1 or 6 of glucose.

Cell extracts of *C. thermocellum* strains LQRI

TABLE 3. *$^{14}CO_2$ formation by C. thermocellum grown on differentially labeled [$^{14}C$]glucose*[a]

| Position of $^{14}C$ in glucose | Sp act[b] (dpm/μmol of $CO_2$) |
|---|---|
| 1-$^{14}C$ | 75 |
| 6-$^{14}C$ | 53 |
| 3,4-$^{14}C$ | 3,200 |
| *U*-$^{14}C$ | 1,066 |

[a] *C. thermocellum* strain LQRI was grown in modified CC medium that contained 0.4% glucose as energy source. The specific activity of the glucose was initially adjusted to 6,400 dpm/μmol in all experiments. After 19 h of growth at 60°C, the specific activity of $CO_2$ in the gas phase was determined. $CO_2$ levels obtained from background growth in the absence of added glucose were subtracted as well as the zero-time levels of radioactive $CO_2$.

[b] Results represent the average of triplicate experiments.

and AS39 contained high activities at 40°C of several glycolytic enzymes, including FDP aldolase (0.39 and 0.77 U/mg) and NAD-specific glyceraldehyde-3-phosphate dehydrogenase (3.67 and 2.83 U/mg, respectively). Neither glucose-6-phosphate dehydrogenase nor gluconate-6-phosphate dehydrogenase was detected, with NADP or NAD as cofactor, in any of the extracts. The glyceraldehyde-3-phosphate dehydrogenase of *T. brockii* was less active (1.73 U/mg at 40°C) than that of cellobiose grown *C. thermocellum* cells.

**Enzymes involved in pyruvate conversion to fermentation products.** The apparent $K_m$ and effect of temperature on the specific activities of lactate dehydrogenase, pyruvate dehydrogenase, hydrogenase, acetate kinase, acetaldehyde dehydrogenase, and alcohol dehydrogenase of the three strains are summarized in Tables 4 and 5. The $Q_{10}$ values for these enzymes varied between 1.5 and 2.7.

The FDP-activated L-lactate dehydrogenases obtained from all three strains were similar in kinetic properties studied, which included the apparent Michaelis-Menten constants for pyruvate and NADH, the pH dependence of FDP activation, the irreversible nature of the enzyme, and the $Q_{10}$. Oxalate ($10^{-5}$ M) was a potent inhibitor, causing 50% inhibition at pH 7.0. Intracellular levels of FDP in exponential-phase cellobiose cultures averaged 18 μmol/g of cells for *T. brockii* and 2.5 and 2.0 μmol/g for *C. thermocellum* LQRI and AS39, respectively.

High clostridial-type phosphoroclastic reaction activities were detected in cell extracts of the three strains and included pyruvate dehydrogenase, hydrogenase, phosphotransacetylase, and acetate kinase. Apparent $K_m$ values measured for these enzymes were in the same range for all strains. Dependence of the pyruvate dehydrogenase reaction on CoA was established and was the basis for measurement of phosphotransacetylase. The latter activity for *T. brockii* was more responsive to the addition of arsenate (2 to 3 mM) than that of LQRI and AS39, which required higher (15 to 20 mM) concentrations. However, equally good activity was observed with phosphate (5 mM), the physiological substrate of the enzyme. Acetate kinase activity in AS39 and LQRI was measured after precipitation of interfering myokinase activity by 60% saturation with $(NH_4)_2SO_4$. Measurement of the reverse reaction for acetyl phosphate formation from acetate was complicated by high adenosine triphosphatase activity in both LQRI (0.8 U/mg) and AS39 (0.45 U/mg). The hydrogenase activity was lowest in *T. brockii* extracts and highest in LQRI extracts.

Acetaldehyde dehydrogenase (CoA acetylating) was detected in extracts of *C. thermocellum* strains and *T. brockii*. Spectrophotometric measurement in both directions was possible with *C. thermocellum* extracts because the alcohol dehydrogenase was inhibited by NAD and did not interfere in the assay. Ethanol dehydrogenases of *C. thermocellum* and *T. brockii* differed significantly. *C. thermocellum* alcohol dehydrogenase (i.e., acetaldehyde reductase) was irreversible in both *C. thermocellum* strains. Only acetaldehyde reduction by NADH was detected and not oxidation of ethanol with NAD. In addition, the alcohol dehydrogenase of *C. thermocellum* was effectively inhibited by low concentrations of the products NAD and ethanol in a competitive manner to the corresponding substrates NADH and acetaldehyde. The apparent $K_i$ for NAD was near $10^{-7}$ M and the apparent $K_m$ for NADH was near $10^{-6}$ M, as based on competitive inhibition experiments. The $K_i$ for ethanol, which was obtained from studies at different acetaldehyde concentrations, was 0.14 mM, a significantly smaller value than the apparent $K_m$ for acetaldehyde (0.5 mM). *T. brockii* extracts contain both a reversible oxygen-labile NAD-linked alcohol dehydrogenase and a reversible NADP-linked alcohol dehydrogenase (9).

**"Ferredoxin" linked enzyme activities.** Experiments designed to identify functional ferredoxin-like proteins in *T. brockii* and in *C. thermocellum* extracts are shown in Table 6. The effect of combining ferredoxins and DEAE-treated extracts from various strains on the CoA-dependent production of acetyl phosphate from pyruvate was tested. The presumptive thermophile "ferredoxins" replaced *C. pasteurianum* ferredoxin in the phosphoroclastic assay system, and the "ferredoxin-free" extracts of *C. thermocellum* recognized pure *C. pasteurianum* ferredoxin. No attempts were made to specifically

TABLE 4. *Effect of temperature on enzymes involved in pyruvate catabolism of T. brockii and C. thermocellum*[a]

| Enzyme | Sp act (μmol/min per mg of protein) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *T. brockii* | | | *C. thermocellum* LQRI | | | *C. thermocellum* AS39 | | |
| | 40°C | 60°C | $Q_{10}$ | 40°C | 60°C | $Q_{10}$ | 40°C | 60°C | $Q_{10}$ |
| L-Lactate dehydrogenase | 0.55 | 1.59 | 1.7 | 0.44 | 2.33 | 2.3 | 0.31 | 1.50 | 2.2 |
| Pyruvate dehydrogenase (CoA acetylating) | 0.53 | 1.19 | 1.5 | 0.48 | 2.12 | 2.1 | 0.60 | 1.94 | 1.8 |
| Hydrogenase (methyl viologen reducing) | 3.3 | 24.0 | 2.7 | 13.0 | — | — | 11.0 | 74.0 | 2.6 |
| Acetate kinase | 1.50 | — | — | 0.78 | — | — | 0.30 | — | — |
| Acetaldehyde dehydrogenase (CoA acetylating) | 0.15 | — | — | 0.35 | — | — | 0.39 | — | — |
| Ethanol dehydrogenase | | | | | | | | | |
| NADH oxidizing | 0.48 | — | — | 0.45 | 2.02 | 2.1 | 0.24 | 1.06 | 2.1 |
| NAD reducing | 0.4 | — | — | <0.005 | <0.005 | — | <0.005 | <0.005 | — |
| NADP reducing | 1.57 | 6.92 | 2.1 | <0.005 | <0.005 | — | <0.005 | <0.005 | — |
| NADPH oxidizing | 1.50 | 6.9 | 2.1 | <0.005 | <0.005 | — | <0.005 | <0.005 | — |

[a] The assay conditions are described in the text. —, Not determined.

TABLE 5. *Substrate concentrations that account for half-maximal velocities of enzyme activities [$(S)_{0.5v, M}$] involved in pyruvate catabolism of T. brockii and C. thermocellum*[a]

| Enzyme | Substrate/activator[b] | $(S)_{0.5v, M}$ | | |
|---|---|---|---|---|
| | | *T. brockii* | *C. thermocellum* | |
| | | | LQRI | AS39 |
| L-Lactate dehydrogenase | Pyruvate | $8.0 \times 10^{-5}$ | $3.5 \times 10^{-5}$ | $4.0 \times 10^{-5}$ |
| | NADH | $1.0 \times 10^{-5}$ | — | $1.5 \times 10^{-5}$ |
| | FDP (pH 6.2) | $5.0 \times 10^{-6}$ | $2.5 \times 10^{-6}$ | $2.5 \times 10^{-6}$ |
| | FDP (pH 7.0) | $2.2 \times 10^{-5}$ | $1.7 \times 10^{-5}$ | $2.2 \times 10^{-5}$ |
| Pyruvate dehydrogenase | Pyruvate | $1.5 \times 10^{-4}$ | $7.5 \times 10^{-4}$ | $1.2 \times 10^{-3}$ |
| Acetate kinase | Acetyl phosphate | $1.0 \times 10^{-4}$ | $4.0 \times 10^{-5}$ | $2 \times 10^{-4}$ |
| Ethanol dehydrogenase | | | | |
| NAD linked | Acetaldehyde | $5 \times 10^{-4}$ | $5 \times 10^{-4}$ | $5 \times 10^{-4}$ |
| | NADH | — | $<1.0 \times 10^{-5}$ | $<1.0 \times 10^{-5}$ |
| NADP linked | Acetaldehyde | $1.5 \times 10^{-4}$ | ND | ND |
| | NADPH | $<1.0 \times 10^{-5}$ | ND | ND |

[a] The activities were quantified at 40°C with the assay conditions described in the text. ND, not detectable; —, not determined.

[b] FDP is the only activator listed.

quantitate activities, and the results shown are only qualitative because aerobic assay procedures were used. It was not possible to demonstrate the interchangeability of ferredoxins in *T. brockii* because DEAE treatment destroyed cell extract enzyme activity. Utilization of *C. pasteurianum* ferredoxin by the phosphoroclastic enzymes of *C. thermocellum* was also demonstrated in experiments in which $H_2$ formation rather than CoA-dependent acetyl phosphate formation was measured. $H_2$ was released at a rate of about 0.1 μmol/min per mg (37°C) without added ferredoxin. This rate was increased threefold by addition of *C. pasteurianum* ferredoxin (0.1 mg/ml).

Table 7 summarizes enzymatic studies on the ferredoxin-associated reduction of pyridine dinucleotides by pyruvate as electron donor. *C. pasteurianum* extracts served as control for ferredoxin NAD(P) oxidoreductase activities (8) in these experiments. The results suggest the presence of high levels of ferredoxin-NAD reductase in *T. brockii* extracts and of ferredoxin-NADP reductase in all three strains. The ferredoxin-NAD(P) reductase activities were not significantly inhibited by the corresponding reduced pyridine nucleotide products (0.2 mM). Low activity of ferredoxin-NAD reductase was also found in *C. thermocellum* strain AS39 but was not detectable in strain LQRI or in the control (*C. pasteurianum*). Increasing the assay temperature from 40°C to 60°C significantly increased

TABLE 6. *Interchangeability of "ferredoxins" from C. thermocellum, T. brockii, and C. pasteurianum in pyruvate catabolism*[a]

| Cell extract source | Ferredoxin added (source and amt) | Net acetyl phosphate formation (μmol) |
|---|---|---|
| *C. pasteurianum* | None | <0.1 |
| | *C. pasteurianum*, 20 μg | 3.4 |
| | *T. brockii*, 0.2 mg | 1.4 |
| | *C. thermocellum*, 0.2 mg | 0.6 |
| *C. thermocellum* | | |
| Strain AS39 | None | 0.7 |
| | *C. pasteurianum*, 100 μg | 2.3 |
| Strain LQRI | None | 0.4 |
| | *C. pasteurianum*, 100 μg | 2.1 |

[a] Reaction mixtures (2 ml) contained 2.5 mg of DEAE-cellulose-treated cell extract protein, 25 mM potassium phosphate, pH 6.3, 0.1 mM CoA, 10 mM pyruvate, and either purified *C. pasteurianum* ferredoxin protein or crude thermophile "ferredoxin" protein. The assay was performed at 40°C.

TABLE 7. *Ferredoxin-NAD(P) reductase activities of T. brockii and C. thermocellum*[a]

| Reaction and conditions | Sp act (U/mg of protein at 40°C) | | | |
|---|---|---|---|---|
| | *T. brockii* | *C. thermocellum* | | *C. pasteurianum* |
| | | LQRI | AS39 | |
| Ferredoxin-NADP reductase | | | | |
| 1. CoA | ND[b] | ND | ND | ND |
| 2. Pyruvate | ND | ND | ND | ND |
| 3. Pyruvate, CoA | 0.18 | 0.03 | 0.03 | 0.02 |
| Ferredoxin-NAD reductase | | | | |
| 1. CoA | ND | ND | ND | ND |
| 2. Pyruvate | ND | ND | ND | ND |
| 3. Pyruvate, CoA | 0.28 | ND | 0.04 | ND |

[a] Reaction mixtures (1 ml) contained 0.25 mg of cell extract protein, 0.1 M Tris-hydrochloride, pH 7.5, 1 mM NADP or NAD, 1 atm of $N_2$, 50 μg of ferredoxin, and, where indicated, 5 mM pyruvate and 0.2 mM CoA.

[b] ND means not detectable; lower limit of assay sensitivity, <0.005 U/mg of protein. *C. pasteurianum* ferredoxin served as ferredoxin source because higher amounts of electron acceptor than present in cell extract are required to demonstrate these activities (8).

the specific enzyme activity, but no additional activities were detected. NADH-ferredoxin reductase activity was detected only in control extracts of *C. pasteurianum* (0.05 U/mg) and not in the thermophilic strains. This activity was dependent on acetyl CoA and excess ferredoxin, as shown previously by Jungermann et al. (8).

A pyridine nucleotide transhydrogenase cycle involving pyruvate, oxalacetate, and malate was suggested in *C. thermocellum* but not *T. brockii* extracts. *C. thermocellum* contained both ammonium-activated NADP-linked malic enzyme (0.91 U/mg in AS39 and 0.23 U/mg in LQRI) and apparently unidirectional NADH-linked oxalacetate reductase (2.7 U/mg in AS39 and 1.5 U/mg in LQRI). Significant levels of these activities were not detected in *T. brockii*. Enzymatic activities associated with the synthesis of oxalacetate from pyruvate or phosphoenolpyruvate were not examined.

## DISCUSSION

These data suggest that the different fermentation product yields observed in different ethanol-producing thermophiles that employ the phosphoroclastic pathway for pyruvate metabolism are related to the specific activities and the direction of specific oxidoreductases that control electron flow (see Fig. 2). For example, cellobiose fermentation balances under identical conditions in *T. brockii* yielded a reduced product ratio for ethanol/$H_2$/lactate of 224:20:352, whereas in *C. thermocellum* strain LQRI the ratio was 157:286:24. In *T. brockii* ethanol yield was higher as a consequence of electron flow from pyruvate to ethanol via pyruvate-ferredoxin reductase, ferredoxin-NAD and -NADP reductases, and both NAD and NADP acetaldehyde reductase. In *C. thermocellum* strain LQRI, ferredoxin-NAD reductase and NADP acetaldehyde reductase were not detectable in cellobiose-fermenting cells. Higher hydrogen yield in LQRI was associated with the absence of detectable electron flow from reduced ferredoxin or NADPH to lactate or ethanol and higher hydrogenase activity. Higher lactate yields in *T. brockii* corresponded with the finding of higher levels of FDP, an allosteric activator of lactate dehydrogenase (4, 22, 23), lower activities of glyceraldehyde-3-phosphate dehydrogenase, and detectable electron flow from pyruvate to NADH via ferredoxin-NAD reductase. The differences in fermentation product yields of *C. thermocellum* strains LQRI and AS39 indicate that metabolic control of electron flow varies in strains and is also greatly influenced by the activity of the pyridine nucleotide oxidoreductases. The in vitro activity of ferredoxin NAD reductase in the three strains examined here was inversely related to hydrogenase activity and in vivo $H_2$ production. Further-

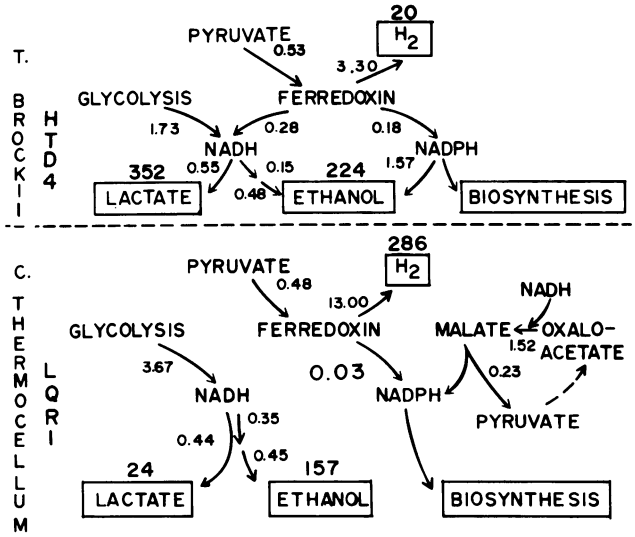


FIG. 2. *Relation of the catabolic enzyme activities and proposed electron flow scheme to the reduced fermentation product yields of T. brockii HTD4 and C. thermocellum LQRI. Numbers represent final end-product yields (total micromoles formed) of cellobiose fermentations and the specific activities of the enzymes indicated by the arrows in micromoles per minute per milligram of protein at 40°C.*

more, the in vivo effect of exogenous hydrogen pressure on the ethanol/acetate ratio during cellobiose fermentation suggests that $H_2$ influences (via interconnected hydrogenase) ferredoxin-NAD oxidoreductase activity in *T. brockii* and *C. thermocellum* strain AS39 but not in strain LQRI. Other metabolic features not examined here may also help explain the different product ratios in these thermophilic strains. For example, the lower lactate yield and intracellular FDP of *C. thermocellum* strains may be related to the specific rate of cellobiose utilization via cellobiose phosphorylase (1). More detailed studies in these thermophiles are required to understand the regulation of catabolic electron flow at the specific enzyme level. Most notably, ferredoxins and NAD(P)-ferredoxin oxidoreductases need to be purified and characterized.

Other properties of pyruvate metabolism suggest that the catabolic enzymes of *T. brockii* and *C. thermocellum* as a unit (i.e., their enzyme outfits) differ significantly. The alcohol dehydrogenase of *C. thermocellum* was active only in the direction of NADH-acetaldehyde reduction. The higher acetate/ethanol ratios of *C. thermocellum* saccharide fermentation as opposed to *T. brockii* may also reflect in part effective inhibition of ethanol dehydrogenase by NAD. On the other hand, *T. brockii* extracts contained reversible NAD- and NADP-linked alcohol dehydrogenase activities (9) that were not effectively inhibited by low NAD(P) or ethanol. The ferredoxin-NADP reductase activity in *T. brockii* is suggested to function in anabolism because of the absence of glucose-6-phosphate dehydrogenase, malic enzyme, and transhydrogenase activity; however, a catabolic role is also implied because of high activity of NADPH-acetaldehyde reductase activity. At present, ferredoxin-NADP reductase activity of *C. thermocellum* extracts can only be suggested to function in anabolism and possibly as a transhydrogenase activity that involves NADPH generation from NADH via oxalacetate reductase and malic enzyme. Recent in vivo studies of Ben-Bassat et al. (unpublished data) have further substantiated the activity and function of various oxidoreductase activities in *T. brockii*. Most notably, *T. brockii* and not *C. thermocellum* grows on ethanol as energy source in co-culture with *Methanobacterium thermoautotrophicum*. In addition, the catabolic function of NADP-linked alcohol dehydrogenase was demonstrated by the reduction of exogenous acetone (recognized by the NAD-linked alcohol dehydrogenase) to isopropanol during glucose fermentation by *T. brockii* cultures.

In general these data substantiate the findings reported in mesophilic clostridia that the function of pyridine nucleotide ferredoxin oxidoreductases depends on both the specific bacterial species and strain and the specific growth conditions of a given strain (6–8). For example, when *C. tyrobutyricum* was grown on glucose, regulation of the NAD-ferredoxin oxidoreductases by NADH and acetyl CoA allowed the

enzymes to function correlatively with glyceraldehyde-3-phosphate dehydrogenase and thus control the levels of NAD and NADH, which influence the flow of electrons from ferredoxin to $H_2$ or butyrate. However, when grown on pyruvate/acetate, NAD-ferredoxin reductase was not detected, and only ferredoxin-NAD reductase controlled the flow of electrons to butyrate. Thus, the catabolic function of NAD-ferredoxin oxidoreductases during saccharide fermentation to ethanol by *T. brockii* and *C. thermocellum* differs from that reported for *C. pasteurianum* and *C. butyricum* (8). Jungermann et al. (8) concluded that these enzymes in mesophilic species functioned in reduction of ferredoxin by NADH in order to explain the high hydrogen fermentation yields. The function of the NAD-ferredoxin oxidoreductase in the thermophilic fermentations described here is in the opposite direction. Namely, the enzyme functions in NAD reduction by ferredoxin, a conclusion that is supported by the measured activity, lack of inhibition by NADH, and the fermentation product yields. Nonetheless, the enzyme probably also functions in the reverse direction when *C. thermocellum* (20) or *T. brockii* (Ben-Bassat et al., unpublished data) are grown in co-culture with *M. thermoautotrophicum* and the only significant reduced end product is methane. It is worth noting here that the ferredoxin ascribed to *C. thermocellum* and *T. brockii* cell extracts remains to be purified, at which time the absolute structural assignment can be documented.

Fundamental understanding of the activity and the direction of oxidoreductases present in the catabolic enzyme outfits of these thermophilic ethanologenic bacteria suggests practical significance including choice of strain, enhancement of ethanol yield, and mutation-selection guidelines for potential industrial strains. Most notably, ethanol yields of *C. thermocellum* and *T. brockii* may be enhanced by obtaining strains via selection-mutation techniques that lack lactate dehydrogenase or hydrogenase, that grow in high (>3%) ethanol concentrations, or that have a combination of these characteristics. In this regard, *C. thermocellum* strain AS39 (not strain LQRI) appears as the strain of choice because the ethanol/$H_2$ fermentation product ratio is higher and a hydrogenaseless mutant should be viable and produce higher ethanol yields because this strain contains ferredoxin-NAD reductase activity. The low ethanol yield of described anaerobic thermophiles may be related to specific regulatory features of their alcohol dehydrogenases, and hence this is a target for further fundamental studies and strain improvement. Enhancement of ethanol yield via metabolic control of a given species' catabolic enzyme outfit (e.g., the ethanol yield increases while the lactate yield decreases as a response to lower intracellular FDP concentration when *T. brockii* is grown on a slowly metabolized substrate) is also of considerable importance and will be discussed in a later paper (Ben Bassat et al., unpublished data).

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